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Topical Review

Basis of Chloride Transport in Ciliary Epithelium

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Abstract. The aqueous humor is formed by the bilayered ciliary epithelium. The pigmented ciliary epithelium (PE) faces the stroma and the nonpigmented ciliary epithelium (NPE) contacts the aqueous humor. Cl^- secretion likely limits the rate of aqueous humor formation. Many transport components underlying Cl^- secretion are known. Cl^- is taken up from the stroma into PE cells by electroneutral transporters, diffuses to the NPE cells through gap junctions and is released largely through Cl^- channels. Recent work suggests that significant Cl^- recycling occurs at both surfaces of the ciliary epithelium, providing the basis for modulation of net secretion. The PE-NPE cell couplet likely forms the fundamental unit of secretion; gap junctions within the PE and NPE cell layers are inadequate to maintain constancy of ionic composition throughout the epithelium under certain conditions. Although many hormones, drugs and signaling cascades are known to have effects, a persuasive model of the regulation of aqueous humor formation has not yet been developed. cAMP likely plays a central role, potentially both enhancing and reducing secretion by actions at both surfaces of the ciliary epithelium. Among other hormone receptors, A_3 adenosine receptors likely alter intraocular pressure by regulating NPE-cell Cl^- channel activity. Recently, functional evidence for the regional variation in ciliary epithelial secretion has been demonstrated; the physiologic and pathophysiologic implications of this regional variation remain to be addressed.

Key words: Aqueous humor inflow — Intraocular pressure — Gap junction — Cl^- channels — cAMP — Adenosine

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Introduction

Secretion of aqueous humor is important, physiologically and pathophysiologically. Aqueous flow provides nutrients and oxygen to the avascular anterior segment and sustains inflation of the globe, ensuring normal visual function in addition to other less well-defined functions (Krupin & Civan, 1995). On the other hand, reducing the secretory rate to lower intraocular pressure (IOP) is a major strategy in treating glaucomatous patients. In principle, reducing the inflow rate might potentially endanger the downstream avascular tissue. Actually, drugs currently used to lower IOP reduce inflow maximally by 25–40%, less than the circadian fall of 50–60% normally observed during sleep (Brubaker, 1998). Therefore, identifying the mechanisms and regulation of aqueous humor formation is highly relevant for developing novel approaches to lower IOP, the only intervention documented to slow the onset and progression of glaucomatous blindness (Collaborative Normal-Tension Glaucoma Study Group, 1998a,b; The AGIS investigators, 2000).

The aqueous humor is secreted by the bilayered ciliary epithelium covering the surface of the ciliary body and comprising the pigmented ciliary epithelial (PE) layer facing the stroma and the nonpigmented ciliary epithelial (NPE) layer facing the aqueous humor (Fig. 1). This bilayered epithelium is unique in orientation. The apical surfaces of the two cell layers are juxtaposed and their basolateral surfaces face outwards. Neighboring cells within and between the layers are coupled by intercellular gap junctions (Raviola & Raviola, 1978). The formation of aqueous humor is driven primarily by active ion secretion across the ciliary epithelium from the stromal to the aqueous-humor surface, followed by osmotic water movement. An alternative view that water might also be stoichiometrically coupled to solute transport through certain symports (Loo et al., 1996; Zeuthen et al., 1997) has been rendered unlikely by recent work (Duquette, Bissonnette & Lapointe, 2001;

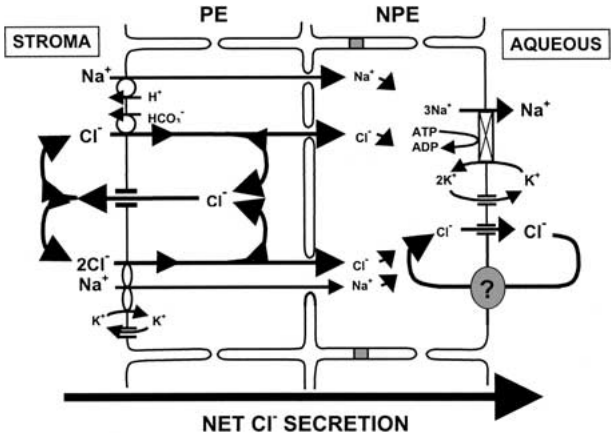

Fig. 1. Partial model of aqueous humor formation. NaCl can be taken up by the PE cells from the stroma by the Na^+-K^+−$2Cl^-$ symport and parallel Na^+/H^+ and $C1^-/HCO_3^-$ antiports, and pass through gap junctions to the NPE cells. Na^+ is extruded by Na^+,K^+-activated ATPase and Cl^- is released through Cl^- channels. Cl^- can also be recycled at both surfaces, through cAMP-activated PE Cl^- channels and through an unidentified mechanism at the aqueous humor surface, possibly a Cl^-/HCO_3^- antiport. Cytosolic carbonic anhydrase CAII enhances turnover of the PE antiports, both by enhancing the rate of delivery of H^+ and HCO_3^- and by directly activating the NHE−1 and AE2 exchangers.

Gagnon et al., 2004). The secretion flows into the posterior chamber of the eye, passes anteriorly between the lens and iris and exits the primate eye largely through the "conventional pathway" consisting of the trabecular meshwork and Schlemm's canal in series into the circulation (Johnson & Erickson, 2000).

The potential importance of anion transfer in forming aqueous humor was early suggested by the polarity of the small open-circuit potential expressed across isolated iris-ciliary bodies of many species. Except for the very earliest studies (Cole, 1961, 1962), investigators have observed that the aqueous surface of the NPE cells is negative to the stromal surface of the PE cells, consistent with a predominant anion secretion from the stroma into the aqueous humor (Holland & Gipson, 1970; Watanabe & Saito, 1978; Kishida et al., 1981; Iizuka et al., 1984; Krupin et al., 1984; Chu, Candia & Podos, 1987; Sears et al., 1991; Do & To, 2000). Attention was initially directed to HCO_3^-, rather than Cl^- transport, for several reasons: (1) HCO_3^- is secreted against a chemical gradient into the aqueous humor in some species, especially the rabbit, a frequently used model for study of aqueous humor dynamics (Davson, 1990); (2) omitting HCO_3^- from the solutions bathing rabbit ciliary epithelium reverses its polarity, so that the transepithelial potential becomes positive (Kishida et al., 1981; Krupin et al., 1984); (3) carbonic anhydrase (CA), an enzyme catalyzing the formation of HCO_3^-, is active in ciliary epithelium (Lütjen-Drecoll & Lonnerholm, 1981; Lütjen-Drecoll, Lonnerholm & Eichhorn, 1983; Wu, Delamere & Pierce, 1997); (4) the CA inhibitor acetazolamide reduces the accessibility of plasma HCO_3^- to the aqueous humor (Maren, 1976); and (5) acetazolamide decreases aqueous humor formation and thereby IOP (Dailey, Brubaker & Bourne, 1982; Rosenberg et al., 1998). The error in this thinking became apparent when it was recognized that humans do not concentrate HCO_3^- but rather Cl^- in the aqueous humor (Davson, 1990), and yet respond to acetazolamide with a reduced inflow rate of aqueous humor (Dailey, Brubaker & Bourne, 1982). Moreover, removal of HCO_3^- does not reverse the negative polarity of the transepithelial potential in the bovine preparation (Do & To, 2000), a species that concentrates Cl^- in its aqueous humor (Gerometta et al., 2003). Until recently, net HCO_3^- transport was not detected across bovine ciliary epithelium (To et al., 2001).

Based largely on work over the past two decades, it is now clear that the mechanisms and regulation of anion transport are indeed important in understanding aqueous humor formation, but Cl^- is the anion of crucial importance (Civan, 1998). Net Cl^- secretion has been measured across the ciliary epithelium of several species (Table 1). HCO_3^- probably plays a significant role in aqueous humor formation, but indirectly by modulating net Cl^- secretion. At present, many of the transport mechanisms underlying Cl^- secretion have been identified, but their regulation and integration are poorly understood. These points and current new directions will be addressed in this review.

Overview of Transepithelial Cl^- Secretion

Figure 1 presents a simplified and abridged summary of the multiple asymmetrically distributed exchangers (antiports), cotransporters (symports), ion and water channels (Verkman, 2003), and H^+- (Wax et al., 1997) and Na^+,K^+-activated ATPases discussed elsewhere (Jacob & Civan, 1996). In essence, net secretion is fundamentally dependent on the dominance of the Na^+,K^+-activated ATPases at the basolateral surfaces of the NPE cells over those of the PE cells (omitted for simplicity). As discussed in detail below, NaCl is taken up from the stroma by electroneutral PE-cell transporters, passes through the gap junctions into the NPE cells, and is released by rheogenic and possibly electroneutral NPE-cell transporters into the aqueous humor.

Cole (Cole, 1961, 1962, 1969) was first to apply the seminal concepts and techniques of Ussing (Ussing & Zerahn, 1951; Koefoed-Johnsen, Ussing & Zerahn, 1952; Koefoed-Johnsen & Ussing, 1958), which initiated the modern era of transport physiology. Thereafter, several investigators demonstrated a clear Cl^- dependence of short-circuit current (I_{sc})

Table 1. Cl^- fluxes across the ciliary body or ciliary epithelial (CE) bilayer under short-circuited condition

Investigators	Year	Species	J_{sa}	J_{as}	Net flux
Holland & Gipson	1970	Cat	12.28	9.39	2.89*
Saito & Watanabe	1979	Toad	7.67	4.12	2.60*
Kishida et al.	1982	Rabbit	15.69	13.44	2.25*
Pesin & Candia	1982	Rabbit	10.9	9.2	1.7
Do & To	2000	Bovine	4.74	3.71	1.03*
Crook et al.	2000	Rabbit CE bilayer	180.3	72.3	108.0*

Flux is expressed as $\mu Eq \cdot h^{-1}\ cm^{-2}$. J_{sa}, stromal-to-aqueous flux; J_{as}, aqueous-to-stromal flux.
*Statistically significant net Cl^- secretion.

across the ciliary body of cat (Holland & Gipson, 1970), toad (Watanabe & Saito, 1978), and rabbit (Kishida et al., 1981). Similarly, substantial net Cl^- transport in the stromal-to-aqueous direction has been demonstrated across the ciliary epithelia of different species (Table 1). In general, these net radiolabeled Cl^- fluxes are at least an order of magnitude larger than calculated values from measured I_{sc} (Kishida et al., 1982; Do & To, 2000). This is consistent with simultaneous net movements of Cl^- and Na^+, whether or not the fluxes are directly coupled through an electroneutral transporter (Pesin & Candia, 1982).

Cl^- Uptake by PE Cells

Cl^- is accumulated intracellularly against an electrochemical gradient (Green et al., 1985; Wiederholt & Zadunaisky, 1986; Carré et al., 1992; Bowler et al., 1996). Based on measurements of intracellular pH (pH_i) and radiolabeled tracer uptake, two major pathways were proposed for Cl^- uptake into PE cells (Wiederholt, Helbig & Korbmacher, 1991): Na^+-K^+-$2Cl^-$ cotransporter and parallel Cl^-/HCO_3^- and Na^+/H^+ exchangers. Results of subsequent work have indicated that both sets of mechanisms are likely operative (Crook et al., 2000; Do & To, 2000; To et al., 2001; Shahidullah et al., 2003), but their quantitative contributions likely vary with species and with physiologic condition within each species.

Na^+-K^+-$2Cl^-$ Cotransporter

As predicted, the Na^+-K^+-$2Cl^-$ cotransporter is immunolocalized primarily along the basolateral border of PE cells in the anterior *pars plicata* region of 1–4 day old calves, the region displaying prominent ciliary processes. The distribution in the calf posterior *pars plana*, the region displaying a relatively flat surface, and the *pars plicata* in adult cows is more complex (Crook et al., 2000; Dunn, Lytle & Crook, 2001). Furthermore, blocking Na^+-K^+-$2Cl^-$ transport activity with the selective inhibitor bumetanide or the less selective inhibitor furosemide has been found to reduce Na^+, Cl^- or water uptake by PE cells or ciliary epithelium under certain experimental conditions. For example, furosemide reduced intracellular Cl^- activity of shark ciliary epithelium (Wiederholt & Zadunaisky, 1986). Bumetanide has been found to inhibit ^{22}Na and ^{36}Cl uptake by cultured bovine PE cells (Helbig et al., 1989) and to shrink native bovine PE cells under isosmotic conditions (Edelman, Sachs & Adorante, 1994). Additionally, reducing the bathing Na^+, K^+, and Cl^- concentrations isosmotically causes shrinkage of PE cells, which is prevented by bumetanide (Edelman et al., 1994). These results demonstrate the presence of a functional, bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter in PE cells.

Blocking the Na^+-K^+-$2Cl^-$ cotransporter with bumetanide also reduces transepithelial transport and aqueous humor formation in vitro. Stromal bumetanide inhibits both I_{sc} and net Cl^- secretion across rabbit (Crook et al., 2000) and bovine (Do & To, 2000) ciliary epithelium, and adding bumetanide to the arterial perfusate slows aqueous humor secretion by isolated bovine eyes (Shahidullah et al., 2003). Interestingly, bumetanide reduces net Cl^- secretion not only by reducing the stromal-to-aqueous Cl^- flux, but also by increasing the aqueous-to-stromal Cl^- transport (Crook et al., 2000; Do & To, 2000). The reasons are not entirely clear, but might reflect two possibilities: (1) enhancement of NPE-cell reabsorptive pathways (Crook, von Brauchitsch & Polansky, 1992; Dong & Delamere, 1994; Civan, Coca-Prados & Peterson-Yantorno, 1996; Matsui et al., 1996; Wu, Pierce & Delamere, 1998; Dunn et al., 2001) triggered by a fall in intracellular Cl^- concentration; the reduction of Cl^- could result if bumetanide is added under conditions where the thermodynamic driving force on the symport favors Na^+-K^+-$2C1^-$ delivery into the cell; and (2) alternatively, bumetanide might enhance leakage through paracellular pathways indirectly.

Parallel $C1^-/HCO_3^-$ and Na^+/H^+ Exchangers

Paired Cl^-/HCO_3^- and Na^+/H^+ antiports are likely important both for regulating pH_i and for Cl^- se-

cretion across ciliary epithelium. This idea was suggested by the observation that Cl^-/HCO_3^- and Na^+/H^+ exchangers are involved in ^{22}Na and ^{36}Cl uptake by cultured bovine PE cells (Helbig et al., 1989; Wiederholt et al., 1991). Further support was provided by measurements of fluid uptake, of ^{22}Na entry and of pH_i in cultured bovine PE cells (Counillon et al., 2000). In the latter study, the pharmacologic profile of Na^+/H^+-exchange inhibition led to identification of the antiport as the NHE-1 isoform. Reverse transcriptase polymerase chain reaction (RT-PCR) amplification of RNA from human ciliary body and immunostaining of the cultured bovine PE cells indicated that the Cl^-/HCO_3^-antiport is the AE2 isoform (Counillon et al., 2000).

The turnover of the antiports is catalyzed by CA (Fig. 1). Its action to accelerate the reversible formation of H^+ and HCO_3^- from CO_2 and water has long been recognized (Meldrun & Roughton, 1933). Thus, cytosolic CAII enhances the delivery of H^+ and HCO_3^- to the antiports. In addition, CAII is now known to bind and directly increase the turnover rates of both NHE-1 (Li et al., 2002) and AE2 exchangers (Sterling, Reithmeier & Casey, 2001), as well. Membrane-bound CAIV catalyzes the reformation of CO_2 and water from H^+ and HCO_3^- at the stromal surface of the PE cells (Wu et al., 1998). It is likely that CA inhibitors reduce inflow by interfering with the actions of CAII and CAIV in catalyzing NaCl uptake by the PE cells.

Relative Importance of Symport and Antiports in Stromal Cl^- Uptake

As noted above, both sets of electroneutral transporters can subserve NaCl uptake by the PE cells from the stroma, but their relative contributions might be different among different species and under different experimental conditions. Measurements of net Cl^- transport across isolated rabbit ciliary epithelia and excised bovine ciliary bodies and of aqueous humor formation by arterially perfused bovine eye preparation suggest approximately equal contributions through the two pathways under certain ambient conditions (Crook et al., 2000; Do & To, 2000; Do, 2002; Shahidullah et al., 2003). However, under other conditions, the parallel antiports may constitute the dominant uptake pathway (McLaughlin et al., 1998). Electron microprobe analyses have demonstrated that: (1) addition of CO_2/HCO_3^- to the external bath increases Cl^- content of the rabbit ciliary epithelium (Bowler et al., 1996; McLaughlin et al., 1998); and (2) in the presence of CO_2/HCO_3^-, either blocking CA with acetazolamide (McLaughlin et al., 1998) or blocking the Na^+/H^+ antiport with dimethylamiloride (McLaughlin et al., 2001b) reduces the Cl^- content, indicating that NaCl is, at least in part, taken up through parallel Cl^-/HCO_3^- and Na^+/H^+ antiports. In the nominal absence of CO_2/HCO_3^-, McLaughlin et al. (1998) found that bumetanide reduced the ciliary epithelial Cl^- content, as expected. However, with CO_2/HCO_3^- present, bumetanide unexpectedly increased the Cl^- content. Under their experimental conditions, the parallel antiports may have increased the cell Cl^- concentration sufficiently high to reverse the thermodynamic driving force imposed on the Na^+-K^+-$2Cl^-$ symport (McLaughlin et al., 2001c), so that blocking the symport could have reduced Cl^- release. The Cl^- concentration at which the thermodynamic driving force would be reversed, favoring release rather than uptake, was calculated to be ~50 mM, close to the estimated baseline Cl^- concentration (Bowler et al., 1996). The increase in Cl^- content might conceivably have also reflected an upregulation of Cl^-/HCO_3^- antiport, overcompensating for the reduction of Cl^- entry into the PE cells when the Na^+-K^+-$2Cl^-$ symport was blocked. However, it seems more likely that under HCO_3^--rich conditions, the symport can be used either to participate in uptake of stromal NaCl or to serve as a release pathway for returning NaCl to the stroma. This interpretation is consistent with the observation that solute transport through the bumetanide-sensitive symport of PE cells can indeed be reversed by reversing the net thermodynamic driving force by reducing bath concentrations of Na^+, K^+ or Cl^- (Edelman et al., 1994).

The potential roles of the Na^+-K^+-$2Cl^-$ symport and parallel Cl^-/HCO_3^- and Na^+/H^+ antiports in aqueous humor formation and IOP modulation have been examined in eyes in vitro and in living animals. Blocking the symport with bumetanide reduced aqueous humor formation by the isolated bovine eye (Shahidullah et al., 2003), but did not affect resistance to outflow or IOP in the living cynomolgus monkey (Gabelt et al., 1997). Also, topical bumetanide did not itself alter IOP in the living mouse (Avila et al., 2002a). However, topical application of each of three selective inhibitors of Na^+/H^+ exchange or of acetazolamide lowered baseline IOP (Avila et al., 2002a). After blocking the Na^+/H^+ antiport, inhibition of Na^+-K^+-$2Cl^-$ cotransporter with bumetanide now significantly reduced IOP. These results are consistent with the view that the paired antiports play a major role in modulating IOP, presumably by affecting inflow, but possibly by altering outflow, as well (Avila et al., 2003).

Recycling of Cl^- at Stromal Surface of PE Cells

PE cells express not only symports and antiports, but also Na^+,K^+-activated ATPase and Cl^- channels at their basolateral surfaces (Jacob & Civan, 1996). The Na^+ pumps and Cl^- channels could subserve Na^+

extrusion and Cl^- release into the stroma, recycling NaCl at the stromal surface of the PE cells and thereby modulating net NaCl secretion into the aqueous humor. This possibility has been supported by electrophysiologic and volumetric measurements of immortalized bovine PE cells (Fleischhauer et al., 2001). Addition of ATP to the bath produced activation of Cl^- channels and thereby cell shrinkage. The ATP likely activated P_2Y_2 ATP receptors (Shahidullah & Wilson, 1997), triggering sequential increases in free intracellular Ca^{2+} concentration, phospholipase A_2 activity, PGE_2 formation and release, and cAMP formation (Fleischhauer et al., 2001). Interestingly, the R_p stereoisomer of 8-bromoadenosine 3′,5′-cyclic monophosphothioate, an inhibitor of cAMP-activated kinase (PKA), mimicked the effects of cAMP, suggesting that cAMP might be acting directly on the Cl^- channel rather than through PKA. The antiestrogen tamoxifen enhances the ATP-induced activation of PE-cell Cl^- channels, but the mechanism is unclear (Mitchell et al., 2000).

The results obtained with transformed PE cells have been confirmed and extended by patch-clamping native bovine PE cells (Do et al., 2004). cAMP was found to activate maxi-Cl^- channels in excised inside-out and outside-out patches. These channels had been identified in many cells, including PE cells (Mitchell, Wang & Jacob, 1997). However, their open probability (P_o) is well known to be highest at a transmembrane potential close to 0 mV, and to decline steeply at increasing polarizations, both positive and negative, so that their physiologic role has been obscure. Interestingly, the P_o of cAMP-activated maxi-Cl^- channels was progressively increased by raising the Cl^- concentration at the cytoplasmic surface from 30 to 65 and 130 mM. These very recent results suggest that the maxi-Cl^- channels might be of particular importance under conditions of rapid NaCl uptake by the PE cells. If the NPE cells could not release NaCl at the same rate into the aqueous humor, the Cl^- concentration would rise within the ciliary epithelium, thereby enhancing Cl^- release through the cAMP-activated maxi-Cl^- channels by increasing P_o (Do et al., 2004). The physiologic trigger for the cAMP production is unknown. However, PE cells are known to release ATP upon cell swelling (Mitchell et al., 1998), possibly triggering the sequential cascade leading to cAMP formation described in the previous paragraph. Thus, PE cells that have taken up large amounts of NaCl and water might display a negative feedback, releasing ATP and initiating the events leading to cAMP-induced Cl^- release back into the stroma. This recycling would reduce net Cl^- delivery to the NPE cells and might even constitute part of a pathway for net reabsorption of aqueous humor.

Cl^- Transfer from PE to NPE Cells

Gap Junctional Transfer of Cl^- From PE to NPE Cells

Gap junctions provide conduits for passage of solutes and water between PE and NPE cells and between adjacent cells within the PE and NPE cell layers, as well. These intercellular junctions have been documented by structural (Reale, 1975; Raviola & Raviola, 1978), biochemical (Coca-Prados et al., 1992; Wolosin, Schütte & Chen, 1997b; Sears, Nakano & Sears, 1998; Coffey et al., 2002) and functional (Green et al., 1985; Wiederholt & Zadunaisky, 1986; Carré et al., 1992; Edelman et al., 1994; Oh et al., 1994; Bowler et al., 1996; Stelling & Jacob, 1997; Do & To, 2000; Do, 2002) studies, leading to the view that the ciliary epithelium is a functional syncytium (Krupin & Civan, 1995). This view was supported by the observation that the commonly used gap-junctional inhibitors octanol (Stelling & Jacob, 1997) and heptanol (Mitchell & Civan, 1997) interrupt communication between fresh bovine PE and NPE cells. Furthermore, heptanol reduces I_{sc} by ~80% across rabbit and bovine ciliary epithelia (Wolosin et al., 1997a; Do & To, 2000). The inhibition of I_{sc} is associated with a reduction in net Cl^- secretion (Do & To, 2000).

In addition to reducing net Cl^- secretion, heptanol causes a concomitant slight decrease in tissue resistance of ~7% (Do & To, 2000; Do, 2002). The effect is reversible upon removal of heptanol from the bathing solution (Do, 2002). Since the transmural conductance (1/resistance) is dominated by the paracellular conductance, it is likely that heptanol exerts at least two effects on ciliary epithelium: interrupting the gap junctions between PE and NPE cells and increasing the permeability of the paracellular pathway. An additional caveat is that there are no uncouplers of gap junctions that are both potent and selective (Rozental, Srinivas & Spray, 1997). Heptanol and octanol are highly potent, but not selective. For example, heptanol triggers slight shrinkage of isolated NPE cells in suspension and also slightly enhances the regulatory volume decrease triggered by cell swelling (Wolosin et al., 1997a).

In view of these caveats, the regulatory role of gap junctions in ciliary epithelial secretion has been recently addressed by electron-probe X-ray microanalyses of the ion contents of PE and NPE cells in the intact rabbit ciliary epithelium (McLaughlin et al., 2004). Under baseline conditions, the Na^+, K^+ and Cl^- contents are similar in neighboring cells within the PE and NPE cell layers and between adjoining PE and NPE cells (Bowler et al., 1996; McLaughlin et al., 1998, 2001c, 2004). In the absence of heptanol, blocking Na^+,K^+-activated ATPase with ouabain on the aqueous, stromal or both sur-

faces produced gains in Na^+ and losses in K^+, which varied considerably among neighboring cells within the PE and NPE cell layers. However, ionic contents of adjacent PE and NPE cells continued to be very similar, whether or not each pair of cells displayed high or normal levels of Na^+ and low or normal levels of K^+. In contrast, with heptanol present, aqueous ouabain produced much larger changes in NPE than in PE cells (McLaughlin et al., 2004).

The electron microprobe analyses demonstrate that the gap junctions linking PE and NPE cells are functionally different from those linking adjacent PE or adjacent NPE cells. This functional difference may reflect at least two factors. First, the PE-NPE gap junctions are much more numerous than those of PE-PE or NPE-NPE junctions (Raviola & Raviola, 1978). Second, the junctions are structurally and possibly functionally different, raising the possibility that ouabain may interrupt PE-PE and NPE-NPE but not PE-NPE junctions. Coffey et al. (2002) have recently identified connexins Cx40 and Cx43 in the PE-NPE gap junctions and connexins Cx26 and Cx31 in the NPE-NPE gap junctions of rat ciliary epithelium. Their immunohistochemical approach was unsuccessful in identifying the connexins in the PE-PE gap junctions, possibly because these connexins are not yet known or because the junctional plaques are very small. Whatever the basis for the greater robustness of the PE-NPE gap junctions, it now appears more appropriate to regard these PE-NPE couplets as the fundamental unit of ciliary epithelial transport, rather than focusing on the ciliary epithelium as a syncytium.

Cl^- Release from NPE Cells into Aqueous Humor

Cl^- Channels

Cl^- efflux from the NPE cells to the aqueous humor is the final step in Cl^- secretion. Three considerations suggest that this Cl^- release likely limits the rate of aqueous humor secretion under physiologic conditions (Jacob & Civan, 1996; Civan et al., 1997): (1) the intracellular Cl^- concentration is several-fold higher than predicted from electrochemical equilibrium, indicating that the uptake of Cl^- from the stroma by the PE cells is not rate-limiting; (2) the membrane potentials and intracellular ionic content of the PE and NPE cells are similar, suggesting an unimpeded ion exchange between PE and NPE cells through the gap junctions; and (3) under baseline conditions, the activities of Na^+,K^+-activated ATPase and K^+ channels at the basolateral membrane of NPE cells are high, indicating that they are not the rate-limiting factors. This thinking is supported by experiments in which addition of the widely used Cl^--channel blocker NPPB (5-Nitro−2-(3-phenylpropylamino)benzoic acid, 0.1 mM) to the aqueous side significantly inhibits both net Cl^- secretion (Do & To, 2000) and aqueous humor formation (Shahidullah et al., 2003) in vitro.

The baseline activity of NPE Cl^- channels can be enhanced by a number of perturbations, including hypotonic swelling of the cells (Yantorno et al., 1992; Zhang & Jacob, 1997), inhibition of PKC activity (Civan et al., 1994b; Coca-Prados et al., 1995; Shi et al., 2002, 2003), stimulation of A_3 adenosine receptors (A_3ARs) (Mitchell et al., 1999; Carré et al., 2000) and introduction of cAMP (Chen et al., 1994; Edelman, Loo & Sachs, 1995; Chen & Sears, 1997). The complexities of regulation are considered under "Regulation of Net Cl^- Secretion" below. The molecular identity of the NPE Cl^- channels is unknown, although at least two different channels are likely expressed (Zhang & Jacob, 1997). Several lines of evidence have suggested that the swelling-activated ClC-3 might be the dominant Cl^- channel in NPE cells (Coca-Prados et al., 1996; Civan, 2003): (1) NPE cells express both ClC-3 transcripts and protein (Coca-Prados et al., 1996; Sánchez-Torres et al., unpublished observation); (2) protein kinase C (PKC) reduces Cl^--channel activity in NPE cells (Civan, Coca-Prados & Peterson-Yantorno, 1994a; Coca-Prados et al., 1995, 1996), a characteristic of ClC−3-associated Cl^- currents (Kawasaki et al., 1994); and (3) antisense deoxynucleotides down-regulate message for ClC-3 and swelling-activated Cl^- channels of NPE cells (Wang, Chen & Jacob, 2000). In addition, blocking antibody specific for ClC-3 (Wang et al., 2003) does reduce Cl^- current of NPE cells (Do & Civan, 2004). Despite these considerations, the role of ClC-3 in Cl^- transport across plasma membranes of NPE and other cells remains controversial (Hermoso et al., 2002; Jentsch et al., 2002), particularly because swelling-activated Cl^--channel activity of mouse pancreatic acinar cells and hepatocytes is retained in ClC-3 negative mice (Stobrawa et al., 2001). One possible interpretation of the conflicting results could be that ClC-3 is of importance in only one of several swelling-activated Cl^- channels expressed in different cells (Yamamoto-Mizuma et al., 2004) and ClC-3 may constitute only part of the complex of proteins comprising a swelling-activated Cl^--channel.

Another protein potentially regulating NPE-cell Cl^--channels is pI_{Cln} (Paulmichl et al., 1992), whose human form was first cloned from NPE cells (Anguíta et al., 1995; Coca-Prados et al., 1995). pI_{Cln} has also been found in native bovine NPE cells (Chen, Wang & Jacob, 1999), and antisense down-regulation of pI_{Cln} reduced the immunological staining and inhibited the swelling-activated Cl^- current. However, pI_{Cln} was later found to be localized in the cytoplasm of NPE cells, and neither a translocation of pI_{Cln} from the cytoplasm to the plasma membrane nor changes in pI_{Cln} expression were detected upon ex-

posing the cells to hypotonic solution (Sánchez-Torres et al., 1999). This suggests that pI_{Cln} might not be on the plasma membrane of NPE cells and that its effect on the Cl^--channel conduit was indirect, possibly through cytoskeletal restructuring.

Electroneutral Transporters

The discrepancy between measurements of I_{sc} and net Cl^- transepithelial transport has raised the possibility that electroneutral transporters might play a role in Cl^- secretion not only at the stromal surface, but at the aqueous surface of the ciliary epithelium, as well (Pesin & Candia, 1982). As noted above, the coupling of Cl^- and Na^+ fluxes need not be direct, but recent work has led to a reexamination of this possibility. Electron microprobe analyses have suggested that the net thermodynamic driving force on the Na^+-K^+-$2Cl^-$ symport of rabbit ciliary epithelium can reverse under certain physiological conditions, favoring release of Cl^- from the PE cells into the stroma and from the NPE cells into the aqueous humor (McLaughlin et al., 1998). Furthermore, several investigators have found Na^+-K^+-$2Cl^-$ symport activity in cultured human (Civan et al., 1996; Crook & Polansky, 1994) and rabbit (Dong & Delamere, 1994) NPE cells. Although the Na^+-K^+-$2Cl^-$ symport may possibly participate in transferring Cl^- from the NPE cells to the aqueous humor (Helbig et al., 1989), recent immunocytochemical studies of young calf ciliary epithelium suggest that the great bulk of the cotransporter associated with the plasma membranes is found at the stromal surface of the PE cells (Dunn et al., 2001).

Recycling of Cl^- at Aqueous Humor Surface of NPE Cells

Volumetric measurements of cultured human NPE cells have demonstrated evidence for functional activity of parallel Na^+/H^+ and Cl^-/HCO_3^- antiports, a Na^+-Cl^- symport and an amiloride-sensitive Na^+ channel (Civan et al., 1996). Message for the α-subunit of the epithelial ENaC Na^+ channel has been confirmed to be present in human ciliary body RNA (Civan et al., 1997). Indirect support for functional Na^+/H^+ and Cl^-/HCO_3^- exchange activity has been provided by the finding of membrane-bound CAIV activity in cultured rabbit NPE cells (Wu et al., 1997) and on the aqueous humor surface of rabbit ciliary epithelium (Matsui et al., 1996). Cl^-/HCO_3^- exchange was also noted in separated NPE layers of rabbit ciliary epithelium (Wolosin et al., 1993).

The physiologic importance of NaCl recycling at the aqueous humor surface is uncertain. However, very recent electron microprobe analyses of intact rabbit ciliary epithelium suggest that a significant fraction of the Na^+ extruded by NPE cells through Na^+,K^+-activated ATPase may actually arise from Na^+ re-entering from the aqueous humor (McLaughlin et al., 2004). Interestingly, volumetric analysis of human NPE cells in suspension has provided some indication that secretion and reabsorption at the aqueous humor surface may be linked and regulated in opposite ways (Civan et al., 1994a, 1996). The kinase inhibitor staurosporine, which has been used to block PKC activity, both inhibits reuptake of fluid by initially shrunken cells (the regulatory volume increase or RVI) and enhances release of fluid by hypotonically swollen cells (the regulatory volume decrease or RVD). Both actions should enhance net secretion. Similarly, the prostaglandin PGE_2 both inhibits the RVI and enhances the RVD (Civan et al., 1994a, 1996), favoring net secretion. The PGE_2-triggered enhancement of the RVD of these cells seems largely mediated by activation of K^+ channels, hyperpolarization of the membranes and increase in the electrical driving force to release Cl^- (Civan et al., 1994a), but may also reflect PGE_2-induced cAMP formation acting to enhance Cl^--channel activity.

Regulation of Net Cl^- Secretion

In contrast to our considerable understanding of the underlying transport components, there is no comprehensive model of the integration and regulation of these components in forming aqueous humor. A substantial literature documents effects of drugs, hormones and signaling cascades, but in general, their full significance is incomplete. Included in this panoply of modulators are the catecholamines, antinatriuretic peptide, endothelin, purines, muscarinic receptors, cannabinoids, tumor necrosis factor-alpha, glucocorticoids, and signaling cascades involving prostanoids, cAMP, cGMP, NO, Ca^{2+}, pH and PKC, PKA and tyrosine and MAP kinases (Chu & Candia, 1985; Chu, Candia & Iizuka, 1986; Civan et al., 1994a; Carré & Civan, 1995; Horio et al., 1996; Crook & Chang, 1997; Brubaker, 1998; Shahidullah & Wilson, 1999; Ellis et al., 2001; Liu, Flammer & Haefliger, 2002; Shi et al., 2002, 2003; Wu et al., 2003; Zhang et al., 2003). In the absence of a comprehensive hypothesis, we shall focus on two of the regulatory pathways that have been examined in considerable detail.

Roles of cAMP

cAMP has arguably been the most intensively studied intracellular modulator of ciliary epithelial secretion. Isoproterenol, a β-adrenergic agonist that increases cAMP production, has been found to activate the Na^+-K^+-$2Cl^-$ symport of PE cells and increase net Cl^- flux across the intact rabbit ciliary epithelium (Crook et al., 2000). As noted above (*v.* Cl^- Release from NPE Cells

into Aqueous Humor), cAMP likely activates some NPE-cell Cl^- channels, as well, so that cAMP might be expected to enhance Cl^- secretion by actions at both surfaces of the ciliary epithelium. However, cAMP does not markedly increase aqueous humor inflow and administration of forskolin to stimulate endogenous cAMP production has actually been reported to decrease inflow (Caprioli et al., 1984; Lee et al., 1984). β-adrenergic-receptor antagonists such as timolol are known to reduce endogenous cAMP production and are clinically used to reduce inflow; whether their inflow effects are solely or primarily mediated by their effects on cAMP remains unproven (McLaughlin et al., 2001b; Yorio, 1985). Interestingly, timolol was found to produce both reduction in intracellular content of K^+ and Cl^- in the intact rabbit epithelium and inhibition of fluid uptake by cultured bovine PE cells, but neither effect was reversed by concomitant application of cAMP (McLaughlin et al., 2001b).

The complexity of the effects produced by forskolin and β-adrenergic-receptor antagonists likely reflects the multiple actions exerted by cAMP on the transporters and channels of ciliary epithelium, which can depend on the routes and concentrations of the drugs applied, the baseline activities of the target components, and also the species studied. Some of the additional actions of cAMP would tend to reduce, rather than increase, net Cl^- secretion. Net Cl^- flux through the PE-cell Na^+-K^+-$2Cl^-$ symport is bidirectional (*v.* Cl^- Uptake by PE Cells), so that symport activation under ambient conditions favoring Cl^- release into the stroma would lead to cAMP-triggered reduction in net secretion. The same result would be produced by cAMP-triggered activation of PE-cell Cl^- channels at the stromal surface (Do et al., 2004). In addition, cAMP possibly uncouples the intercellular gap junctions between PE and NPE cells (Do, Kong & To, 2004). Finally, cAMP has been reported to inhibit Na^+,K^+-activated ATPase activity in NPE cells (Delamere & King, 1992; Nakai et al., 1999), which would reduce Cl^- secretion. These effects may have played a role in the observed forskolin-triggered decrease in inflow (Caprioli et al., 1984; Lee et ah, 1984).

Given this broad spectrum of actions, it seems improbable that the integrated response of cells, tissues and organs could be determined by the average intracellular cAMP concentration. Indeed, Huang et al. (2001) have elegantly demonstrated the role of cAMP compartmentalization. At 1 μM, adenosine did not significantly alter the total cell cAMP concentration and yet elevated the local cAMP concentration to activate CFTR Cl^- channels of Calu-3 cells. Evidently, a full understanding of the regulatory role of cAMP in ciliary epithelial secretion will require a mapping of the time course of cAMP concentration in the microenvironments of the underlying transporters.

Role of A_3 Adenosine Receptors (A_3ARs)

Among many other potentially important regulators of aqueous humor inflow noted above, adenosine receptors are of particular interest because knockout of A_3ARs has recently been found to reduce IOP in the living mouse (Avila, Stone & Civan, 2002b). Adenosine can be physiologically delivered to the two surfaces of the ciliary epithelium by ATP release from both PE and NPE cells and subsequent ecto-enzymatic conversion (Mitchell et al., 1998).

Adenosine has been reported to stimulate whole-cell Cl^- currents of freshly harvested bovine NPE cells (Carré et al., 1997). A_3AR-selective agonists also activated whole-cell Cl^- currents of cultured human NPE cells, and that activation was precluded by a selective A_3AR antagonist (Carré et al., 2000). The effects of purinergic agents on cell volume have also been studied with cultured human NPE cells in suspension. Under conditions where Cl^--channel activity limited cell shrinkage, A_3AR-selective agonists stimulated shrinkage and this effect was blocked by A_3AR-selective antagonists (Mitchell et al., 1999). Additionally, shrinkage triggered by the non-selective P-1 agonist adenosine was blocked by the A_3AR-selective antagonists (Mitchell et al., 1999). Transcripts for A_3ARs were found expressed by the cultured human cells and rabbit ciliary processes (Mitchell et al., 1999).

These in vitro results have been extended to the living mouse. As predicted, A_3AR-selective agonists increased IOP and A_3AR-selective antagonists both reduced IOP and markedly inhibited the subsequent response to adenosine (Avila, Stone & Civan, 2001; Avila et al., 2002b). The effects did not reflect cross-over actions on other P-1 receptors. A_3-knockout (*Adora*$3^{-/-}$) mice displayed a reduced baseline IOP and reduced responses to adenosine, an A_3AR-selective agonist and an A_3AR-selective antagonist (Avila et al., 2002b). Despite the consistency of the in vitro and in vivo results, there is substantial species variation in A_3 receptors (Jacobson et al., 1997; Linden, 2001) so that the full implications of these results for humans are as yet unclear. Interestingly, A_3ARs may play a role in the pathogenesis of, or defensive responses to, certain forms of glaucoma since these receptors are overexpressed by an order of magnitude in NPE and outflow pathway cells in the pseudoexfoliation syndrome, a major cause of open-angle glaucoma (Schloetzer-Schrehardt et al., 2004).

Topology of Net Cl^- Secretion

The cartoon of Fig. 1 might suggest that secretory activity is constant across the entire ciliary epithelial surface. However, observations obtained in several different mammalian species with histochemical approaches (Flügel & Lütjen-Drecoll, 1988; Flügel

et al., 1989, 1993; Eichhorn, Flügel & Lütjen-Drecoll, 1990; Eichhorn & Lütjen-Drecoll, 1993), immunocytochemistry (Dunn et al., 2001) and molecular probes (Ghosh et al., 1990, 1991) have indicated regional differences in the expression of Na^+,K^+-activated ATPases and other proteins and biologically active peptides. The isozymes of Na^+,K^+-activated ATPase display differences in both function and hormonal regulation (Sweadner, 1989; Gao et al., 2002; Lingrel et al., 2003). In principle, regional specificity of the isozymes and their regulators (Feschenko et al., 2003) could lead to regional differences (particularly antero-posteriorly) in net secretion by these areas (Ghosh et al., 1990, 1991). However, the functional topography of the ciliary epithelium has resisted investigation because of its structural complexity. Recently, a beginning has been made in addressing this issue by in vitro electron probe X-ray microanalysis of the ionic contents of rabbit ciliary epithelium. Three different experimental strategies for altering intracellular Cl^- content led to greater changes in the anterior than in the posterior ciliary epithelium (McLaughlin, Macknight & Civan, 2001a). More recently, application of ouabain to block Na^+,K^+-activated ATPase produced faster loss of intracellular K^+ and gain of intracellular Na^+ and at lower concentration anteriorly than posteriorly (McLaughlin et al., 2004). The results of the two electron microprobe studies strongly suggest that turnover of Na^+, K^+ and Cl^- is far greater in the anterior than in the posterior ciliary epithelium. The full implications of these findings in expressing regional differences in net aqueous humor formation remain to be determined.

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